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Meeting report

The greening of virology: Plant Virology Satellite Symposium, American Society for Virology, UC-Davis, July 12, 2003

Theo W. Dreher[a,*] and A.L.N. Rao[b]

[a] *Department of Microbiology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331, USA*
[b] *Department of Plant Pathology, University of California, Riverside, CA 92521-0122, USA*

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The American Society for Virology (ASV) annual meeting serves as a yearly gathering point for U.S. and international scientists conducting molecular studies on plant viruses. This meeting has recently been enhanced by the inclusion of a satellite symposium highlighting advances in plant virology that precedes the formal meeting. This year's day-long symposium, held on July 12, 2003 and attended by some 200 virologists, covered host defense mechanisms, novel insights into viral symptom induction, virus replication and movement, and the use of plants to produce edible vaccines.

The symposium began with a lecture by Dr. George Bruening (UC-Davis), who reviewed his laboratory's studies of the 359-nt satellite (sat) RNA of *Tobacco ringspot virus*. Infected tissue accumulates monomeric and oligomeric linear and circular forms of both plus and minus polarities, with linear molecules being encapsidated. The circular forms suggested rolling circle transcription for both polarities of the RNA, with cleavage of oligomeric transcripts to the unit length and ligation to form circles as other steps in the replication scheme. Ligation of plus polarity RNA was not observed in vitro and was studied in vivo by expression from a geminivirus (single-stranded DNA virus) vector. On the other hand, ligation of minus RNA and cleavage of both polarities of oligomeric RNA were observed in vitro. Furthermore, these steps were shown to be RNA-mediated, with no protein requirement. Mutational analyses revealed that a few dozen nucleotides are sufficient for each reaction. These sequences are sources of the hammerhead and hairpin ribozymes now being applied in gene regulation and antiviral strategies in animal and plant systems. This is an excellent example of an advance that originated in plant virology but is now widely applied in many areas of research and biotechnology.

RNA interference (RNAi) and its relationship to plant virus infections has over the last few years been one of the most rapidly developing fields in plant virology and was a focus of this year's symposium. Dr. József Burgyán (Agricultural Biotechnology Center, Gödöllö, Hungary) presented the results of a survey of cloned 21-nt-long interfering RNAs (siRNAs) present in plants infected with *Cymbidium ringspot tombusvirus*. Most of the siRNAs (80%) were derived from the positive-strand viral RNA, with a distribution that did not randomly represent the entirety of the viral RNA. This led to the provocative suggestion that siRNAs in this system predominantly originate by direct Dicer (the ribonuclease responsible for the generation of siRNAs) cleavage of imperfect duplexes in the most folded regions of the positive-sense genomic RNA. This is analogous to the production of miRNAs and contrasts with other recent interpretations in which siRNAs arise from double-stranded RNA that is either produced by viral transcription events or by the action of host RNA-dependent RNA polymerase. Dr. Burgyán also described studies on the properties of p19, the viral protein that suppresses the silencing response of the host. Biochemical and X-ray crystallographic experiments indicate that p19 binds perfect and near-perfect 21-nt siRNA duplexes. This sequesters the RNAs that are central to the silencing response, as indicated by the observation that almost all siRNAs present in infected plants were found in p19–siRNA complexes.

Dr. Hong-Wei Li (laboratory of Dr. Show-Wei Ding, UC-Riverside) described the application of experience gained with plant viruses to uncover virus-induced gene silencing and the existence of a viral silencing suppressor in

* Corresponding author. Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, OR 97331-3804, USA. Fax: +1-541-737-0496.

E-mail address: theo.dreher@orst.edu (T.W. Dreher) .

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an animal (insect) virus infection. *Flock house alphanodavirus*, which can replicate in plant and animal cells, induces strong RNA silencing and encodes a silencing suppressor (protein B2) as demonstrated using indicator plants designed to detect RNAi suppressor activity. Induction of virus-specific siRNAs and RNAi suppression were also demonstrated in *Drosophila* cells. Viral replication in these cells requires B2 expression, but B2 is superfluous if the host RNAi response is compromised by depletion of AGO2 expression, which is needed for siRNA-targeted RNA degradation. Virus-induced silencing and suppression of silencing are also hallmarks of infections by *Nodamura alphanodavirus*, which infects both *Drosophila* and *Anopheles* mosquito cells. Dr. Li's studies raise the possibility that RNAi is a common component of the defense response of animal cells to viral infection.

In studies that have paralleled those unraveling the role of RNA silencing in virus infections, it is now clear that microRNAs (miRNAs) produced from nuclear genes are controllers of many gene families, particularly those involved in developmental pathways. The proteins that produce, target, and carry out the functions of miRNAs and virus-induced siRNAs are the same or closely related. It is thus in retrospect not surprising that viral suppressors of silencing impair the normal function of miRNAs, by inhibiting miRNA-guided degradation of target mRNAs. Studies reported by Dr. Kristin Kasschau (laboratory of Dr. James Carrington, Oregon State University) have cataloged miRNAs (20–21 nt) produced in *Arabidopsis* that target certain mRNAs for degradation, such as those encoding transcription factors involved in tissue development and hormone response, and proteins of the RNA silencing pathway itself. Transgenic *Arabidopsis* plants expressing the silencing suppressor from *Turnip mosaic potyvirus* (P1/HC-Pro) display phenotypes that are characteristic of infected plants (e.g., stunting, leaf curling, flower abnormalities). Similar phenotypes result from mutational inactivation of the RNaseIII domain-containing protein responsible for the generation of miRNAs in plants, DICER-LIKE1 (DCL1). These studies indicate that many virus-induced symptoms in plants are, at least partly, the result of viral RNA silencing suppressors affecting miRNA-controlled pathways, rather than the result of general dysfunction caused by cells exhausted from producing large quantities of virus.

A different type of symptom induced by some virus–host combinations is necrosis. A lethal tomato disease involving necrosis is caused by infection with *Cucumber mosaic virus* together with D satRNA (Dr. Ping Xu, laboratory of Dr. Marilyn Roossinck, Noble Foundation, Ardmore, OK). Microscopic and ultrastructural observation of the developing necrosis and observation of DNA ladders upon electrophoresis of nuclear DNA indicate the involvement of apoptosis or programmed cell death. By simultaneous in situ hybridization to detect satRNA and staining to detect nuclear DNA fragmentation, cell death was observed to begin in satRNA-infected developing phloem and cambium cells and to quickly spread to adjacent infected cells. Inoculation of tomato mutants indicated roles for ethylene and jasmonic acid, but not salicylic acid, signaling in the necrotic response. No natural resistance to this disease has been found in tomato, but a recessive tolerance occurs in the close relative *Solanum lycopersicoides*. Introgressed tomato/*S. lycopersicoides* hybrids are being tested in an effort to isolate the "disease gene" in tomato that interacts with the satRNA to induce programmed cell death.

Another virus whose symptoms can be intensified by coinfection with a satRNA is *Turnip crinkle virus*. Dr. Anne Simon (University of Maryland) reported that virion levels are substantially reduced in the presence of satC RNA. Interestingly, infection with a mutant virus whose coat protein has a short N-terminal insertion resulted in low virion levels and enhanced symptoms characteristic of coinfection of wild-type TCV with satC. Apparently, satC interferes with encapsidation, increasing the levels of free coat protein, which serves as a suppressor of RNA silencing. Intensified symptoms could result from the consequent increased viral replication and spread, and perhaps from interference with host miRNA function. To visualize viral colonization of the plant, a technique developed for yeast studies has been adapted. The presence in the cytoplasm of viral RNAs tagged with the coat protein binding site of phage MS2 causes relocalization of constitutively expressed CP_{MS2}-NLS-GFP fusion protein from the nucleus. Initial results have indicated that viral movement is indeed more extensive in the presence of satC.

A notable characteristic of plant virus infections is that they do not involve extracellular phases within the host, but rather spread via the plasmodesmatal connections between cells. These connections permit exchanges of small molecules but normally are a barrier to macromolecules as large as viruses or their genomes. As a result, movement proteins, which permit viral spread through plasmodesmata, are critical components in the arsenals of plant viruses. Dr. Maria Rojas (laboratory of Dr. Robert Gilbertson, UC-Davis) reported on the movement proteins of two viruses from the genus *Begomovirus*. These are whitefly-transmitted geminiviruses whose genomes consist of either one or two circular single-stranded DNAs. The contributions of viral proteins to virus movement were probed by microinjection of fluorescently labeled viral protein and/or DNA in protoplasts and epidermal cells, expression of proteins in transgenic plants, and in vitro DNA-binding analyses. For *Bean dwarf mosaic virus*, which has a bipartite genome and is not phloem-limited, the proteins BV1 and BC1 coordinate the movement of ss- and/or ds-DNA across the nuclear and plasmodesmatal boundaries, respectively, and the capsid protein is dispensable for cell-to-cell and long-distance movement. For *Tomato yellow leaf curl virus*, which has a monopartite genome and is phloem-limited, the capsid protein plays the role of BV1 in mediating nucleocytoplasmic transport. Cell-to-cell movement was observed to be limited to the phloem, requiring two proteins, V1 and C4, neither of which is a

homolog of BC1. The catalog of plant virus movement proteins is indeed varied and large.

Plant virology has at its disposal a range of sophisticated techniques that allow rapid dissection of gene function, even for viruses with large gene complements and big genomes. Two presentations described such investigations using members of the *Closteroviridae*, positive-strand RNA viruses with genomes about 15 kb long. Dr. Valerian Dolja (Oregon State University) described *Beet yellows closterovirus*, whose monopartite genome encodes 10 proteins. Functions have now been associated with each of these proteins using reverse genetics and agroinfection techniques. RNA amplification requires two core replicase proteins and two replicational enhancers, the leader proteinase and a suppressor of RNA silencing. A 6-kDa integral membrane protein serves as the viral movement protein. The remaining five proteins are required for assembly and transport of the long, filamentous virions. Four of these are associated with the tail at one end of the virion, which appears to function as a mediator of cell-to-cell and long distance movement. The unique Hsp70 (chaperone) homolog, which appears to interact with the cytoskeleton, is one of the tail proteins.

Dr. Bryce Falk (UC-Davis) described *Lettuce infectious yellows virus* (LIYV) of the genus *Crinivirus*, all of whose members have emerged in the last 20 or so years. Criniviruses are economically important phloem-limited viruses with a bipartite genome and are transmitted by whiteflies. These viruses have gene complements that are clearly related to those of their monopartite closterovirus relatives, but they also have some unique genes. P34, a protein with RNA-binding activity that is encoded by ORF2 of LIYV RNA1, is not needed for RNA1 replication, but is required for efficient *trans*-accumulation of RNA2. P26 is encoded by ORF7 of RNA2, and unlike most of the proteins encoded by RNA2, is not a component of the complex virion. It is not an essential protein, but is abundant, and ultrastructural studies indicate a localization that is associated with unique cytopathological structures termed plasmalemma deposits. Virions are often associated with these features, but their function is as yet unknown.

Proper maintenance of genome termini is critical for viruses with linear genomes. For *Brome mosaic virus*, three factors contribute to 3′-end integrity: repair by CCA nucleotidyltransferase, priming with RNA fragments made by abortive initiation, and recombination resulting from template switching by the replicase (Dr. Cheng Kao, Texas A&M University). Use of chemically synthesized templates with modified nucleotide functional groups and lengths of the initiation sequence have revealed defined requirements for initiation and terminus maintenance by BMV replicase.

Dr. Hélène Sanfaçon (Pacific Agri-Food Research Center, Summerland, BC, Canada) addressed the connection between RNA replication and membranes in infected cells. *Tomato ringspot nepovirus* has a bipartite positive-strand RNA genome with a 5′-VPg. The putative viral NTP-binding protein NTB is an integral membrane protein that is associated together with an NTB-VPg precursor protein in cell extracts containing ER-derived membranes that have replicase activity. Analysis of protein/membrane topology after in vitro translation with canine microsomes and from studies with infected cell extracts indicate that the VPg domain of NTB-VPg is in the ER lumen, inaccessible from the cytoplasmic site of its presumed role in priming RNA replication. Cytoplasmic VPg may be derived from other less abundant precursor forms of VPg (e.g., VPg-Pro-Pol) present in infected cells. The sites of RNA replication may assemble via protein–protein interactions between cytoplasmic VPg and Pol domains and the putative membrane anchor NTB-VPg.

In the final talk of the symposium, Dr. Hilary Koprowski (Thomas Jefferson University, Philadelphia) described the use of plant virus vectors for the production in plants of cheap, readily accessible vaccines against human and animal infectious diseases. Such ingested vaccines could make vaccination universally available for the first time because of the ease of production, dissemination, and administration, as well as the projected low costs. Vaccine antigens have been produced from recombinant *Alfalfa mosaic virus* (AMV) in which the coat protein gene has been replaced with antigen-coding sequences, or from recombinant *Tobacco mosaic virus* genomes that express fusions of antigen with the AMV coat protein. Animal tests have shown protection against *Respiratory syncytial virus*. In another approach, antibodies reacting with rabies virus and human colorectal antigen have been produced in transgenic tobacco using constructs that include an AMV translational enhancer. Vaccines against rabies, HIV, anthrax, colorectal cancer, and other disease-causing organisms are being developed.

The presentations briefly described here undoubtedly stirred participants' imaginations to devise ways to delve deeper into the inner workings of plant viruses. At next year's Plant Virology Satellite Symposium in Montreal we hope to hear the results of some of the ideas hatched during these stimulating talks.